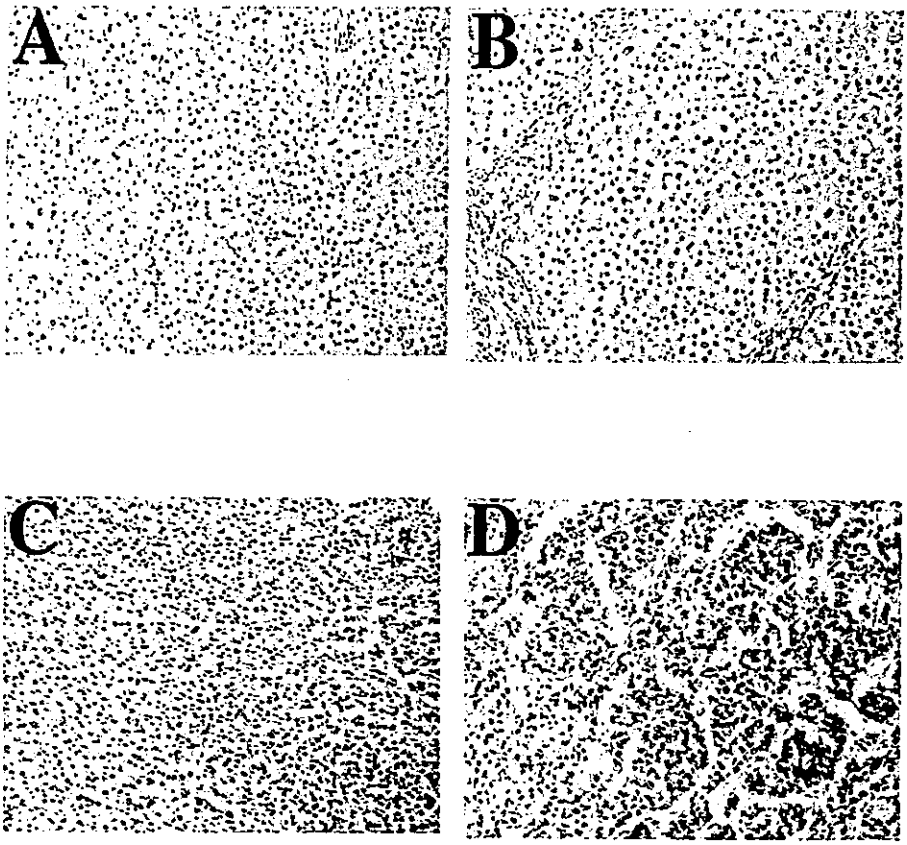

Figure 1. Immunohistochemical staining of Akt1. Akt1 protein was faintly stained in the cytoplasm of both cancer and non-cancer cells. No Akt1 staining was observed when the primary antibody was substituted with PBS. A, Chronic hepatitis; B, Liver cirrhosis; C, Well-differentiated HCC; D, Poorly-differentiated HCC. Magnification x100.

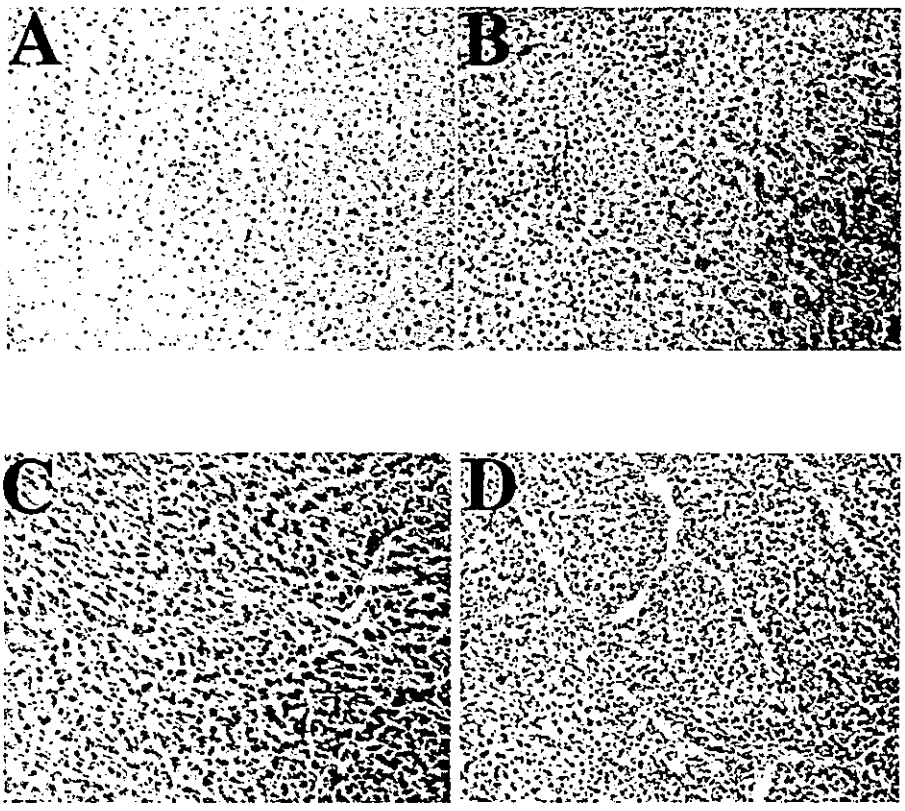

Figure 2. Immunohistochemical staining of Akt2. Akt2 was stained in the cytoplasm of both cancer and non-cancer cells with a broad spectrum. No Akt2 staining was observed when the primary antibody was substituted with PBS. A, Chronic hepatitis; B, Liver cirrhosis; C, Well-differentiated HCC; D, Poorly-differentiated HCC. Magnification x100.

Table I. Immunohistochemical analysis of Akt1 and Akt2 protein in cancer and non-cancer tissues.

	No. of cases	Intensity (Akt1)		Intensity (Akt2)	
		Low	High	Low	High
Normal liver	4	3	1	4	0
Chronic hepatitis	23	18	5	16	7
Cirrhosis	29	19	10	3	26
HCC	56	42	14	35	21
Well-differentiated	6	4	2	0	6
Moderately-differentiated	18	17	1	11	7
Poorly-differentiated	32	21	11	24	8

[a]p<0.001; [b]p<0.05.

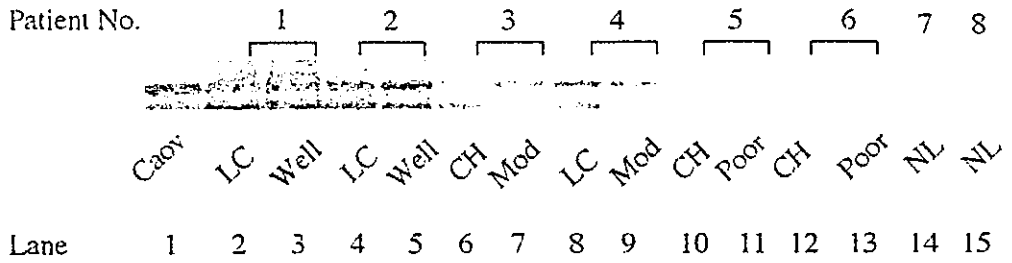

Figure 3. Western blotting using anti-human Akt2 antibody. Six paired non-tumor and tumor tissues and 2 normal livers were examined for Akt2 expression. The lysate from CaoV cells (30), an ovarian cancer cell line, yielded a 56,000 Da band of Akt2 protein (lane 1). When pre-absorbed antibody was applied to the transferred membrane instead of the Akt2 antibody, no bands appeared on the blots (data not shown). Akt2 was not detected in 2 normal liver tissues (lanes 14 and 15). Non-cancer tissues with chronic hepatitis expressed only faint or undetectable Akt2 (lanes 6, 10 and 12), while 3 cirrhosis tissue samples expressed moderate to high levels of Akt2 protein (lanes 2, 4 and 8). Well-differentiated HCC expressed a high level of Akt2 (lanes 3 and 5). Moderately-differentiated HCCs showed moderate bands (lanes 7 and 9) while poorly-differentiated HCCs did not express Akt2 protein (lanes 11and 13).

expression (grade 1), and the remaining 40 tissues had no detectable Akt1 expression (grade 0), as shown in Table I. There was no significant relationship between histological grade of hepatitis and Akt1 expression. In HCC tissues, 14 patients (25%) exhibited high Akt1 expression. Again, there was no significant relationship between HCC-related histopathological parameters and Akt1 expression.

Akt2 protein was also stained in the cytoplasm of both cancer and non-cancer cells. However, the expression of Akt2 protein showed a wide spectrum of staining in contrast to Akt1 protein expression. In normal liver and chronic hepatitis, Akt2 protein expression was generally low (grade 0 or 1, as shown in Fig. 2A). However, the majority of cirrhotic livers showed marked Akt2 expression, which was particularly intense in regenerative nodules (Fig. 2B). The high expression of Akt2 protein (90%, 26/29) was significantly more frequent in liver cirrhosis compared with normal liver (0%, 0/4), chronic hepatitis (30%, 7/23) and cancer tissues (38%, 21/56), as shown in Table I. In cancer tissues, the histopathological type of HCC was closely correlated with Akt2 expression, which decreased according to cell differentiation. All well-differentiated HCCs exhibited high Akt2 expression while poorly-differentiated HCCs tended to exhibit low Akt2 expression (Table 1, Fig. 2C and D). High expression of Akt2 was more frequent in well-differentiated HCC than in normal liver, chronic hepatitis or less differentiated HCCs.

Western blot analysis for Akt2. Since the expression of Akt2 protein, but not Akt1, correlated with histopathological findings of hepatitis or HCC, Western blot analysis with anti-Akt2 antibody was performed in the 8 patients to further confirm the expression of Akt2 protein (Fig. 3). Consistent with the results of immunohistochemical staining, Akt2 expression was not detected in 2 normal livers (lanes 14 and 15). Akt2 protein expression ranged from faint to moderate in chronic hepatitis (lanes 6, 10 and 12), and was more intense in liver cirrhosis (lanes 2, 4 and 8). In HCC tissues, Akt2 protein was markedly expressed in well-differentiated HCC (lanes 3 and 5), but less in moderately-differentiated HCCs (lanes 7 and 9). In poorly-differentiated HCCs (lanes 11 and 13), Akt2 protein expression was barely detectable.

Correlation between Akt protein expression and clinicopathological parameters. Clinicopathological parameters were compared between high and low Akt protein expression groups (Table II). A significant difference was observed in Akt2 protein expression in association with histopathological differentiation, portal vein tumor thrombus, and number of tumors, while no such difference was observed with Akt1 protein expression. High Akt2 protein expression was associated with more differentiated carcinomas (p=0.026), whereas low expression of Akt2 protein was associated with portal vein tumor thrombus (p=0.023) and multiple intra-

Table II. Relationship between Akt expression and clinicopathological parameters in HCC.

		Akt1			Akt2		
	No. of cases	Low	High	p-value	Low	High	p-value
Age							
≥60	31	23	8	NS	21	10	NS
<60	25	19	6		14	11	
Gender							
Male	43	33	10	NS	28	15	NS
Female	13	9	4		7	6	
Tumor size							
<2	19	12	7	NS	11	8	NS
≥2	37	30	7		24	13	
Histologic differentiation							
Well/moderately-differentiated	24	21	3	NS	11	13	0.026[a]
Poorly-/undifferentiated	32	21	11		24	8	
Portal vein tumor thrombus (Vp)							
Yes	8	5	3	NS	8	0	0.023[a]
No	48	37	11		27	21	
Hepatic vein invasion (Vv)							
Yes	3	2	1	NS	3	0	NS
No	53	40	13		32	21	
No. of tumors							
Solitary	26	16	10	NS	24	2	0.001[a]
Multible	30	26	4		11	19	
Septal formation (Sf)							
Yes	27	22	5	NS	17	10	NS
No	29	20	9		18	11	
Capsular formation (Fc)							
Yes	42	36	6	NS	25	17	NS
No	14	6	8		10	4	
Capsular invasion							
Yes	22	18	4	NS	15	7	NS
No	20	18	2		10	10	

NS, not significant. [a]Statistically significant.

hepatic tumors (p=0.001). There was no relationship between the expression of these 2 proteins and the other clinicopathological parameters listed in Table II.

Correlation between Akt protein expression and survival. Disease-free survival in HCC was significantly better in patients with high Akt2 protein expression, compared to those with low Akt2 protein expression (p<0.001, Fig. 4). No such difference was observed with Akt1 protein expression. Likewise, a significantly better survival rate was observed in the high Akt2 expression group compared with the low Akt2 expression group (p=0.038), but no such difference in survival rate was observed with Akt1 protein expression (Fig. 5).

Univariate and multivariate analyses of prognostic factors. Among the parameters investigated (Table II), high Akt2 protein expression, histological differentiation and portal vein invasion were found to be significant prognostic factors by univariate analysis (p=0.001, 0.028, and 0.006, respectively). In the multivariate analysis (Table III), Akt2 protein expression and histological differentiation were found to be significant covariates (relative risk, 6.4-fold, p=0.012, and 3.9-fold, p=0.048, respectively).

Discussion

The main finding of the present study was that Akt2 expression in HCC, but not Akt1, was significantly associated with

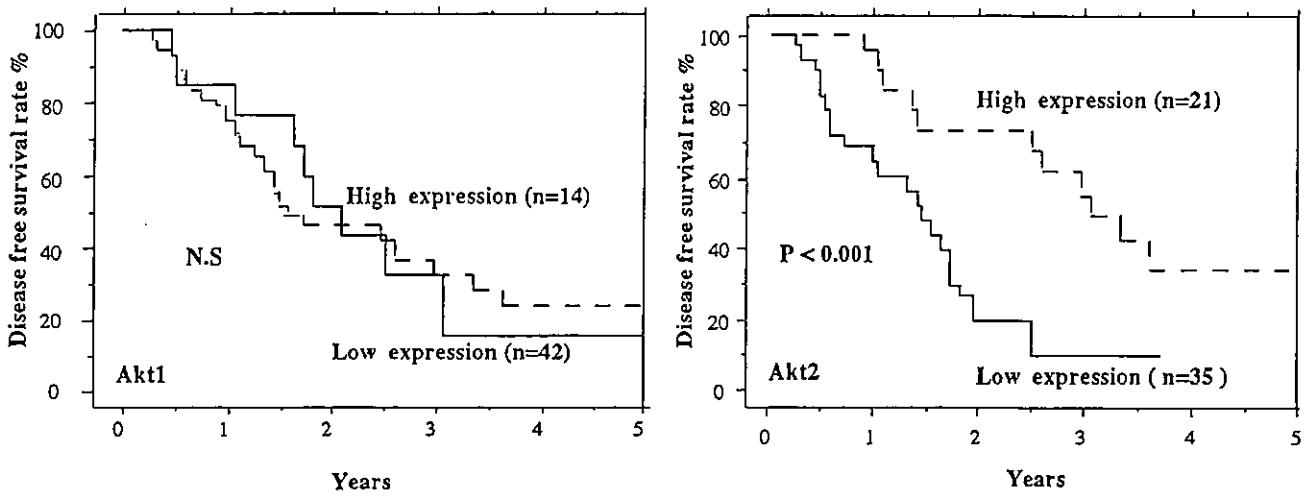

Figure 4. Kaplan-Meier curves for disease-free survival according to Akt1/Akt2 expression in 56 patients with HCC. Patients with low Akt2 expression had a significantly worse prognosis than those with high Akt2 expression ($p<0.001$). Such a relationship was not observed with Akt1 expression.

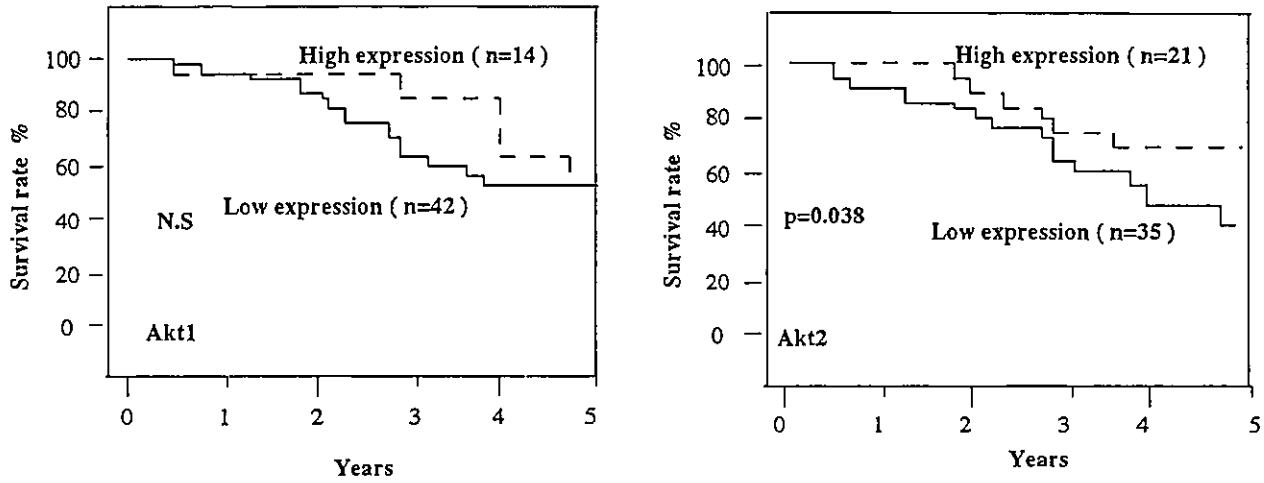

Figure 5. Kaplan-Meier curves for overall survival rate according to Akt1/Akt2 expression in 56 patients with HCC. Patients with high levels of Akt2 protein had a significantly better survival than those with low level Akt2 expression ($p=0.038$). Expression levels of Akt1 were not significant.

Table III. Univariate and multivariate analysis (Cox proportional hazards model).

	Univariate	Multivariate		
Categories	p-value	p-value	Risk ratio	95% Confidence interval
Akt2 (high:low)	0.001	0.012	6.351	1.276-7.017
Differentiation (well/mod.:poor/undiff.)	0.028	0.048	3.904	0.223-0.994
Portal vein invasion (yes:no)	0.006	0.420	0.651	0.235-1.828
No. of tumors (solitary:multible)	0.081	0.288	1.130	0.259-1.193

Well/mod., well/moderately-differentiated; poor/undiff., poorly-/undifferentiated.

prognosis of patients with HCC. Indeed, both disease-free survival rate and overall survival rate were significantly better in patients with high Akt2 expression than in those with low Akt2 expression (Figs. 4 and 5). The significance of Akt2 protein as a prognostic factor was also confirmed by multivariate analysis (Table III). These findings may be explained by the histopathological results, which revealed that prognostic factors such as cell differentiation, portal vein

involvement and number of tumors were significantly less frequent in patients with high Akt2 expression compared to those with low Akt2 expression (Table II). In addition, the risk ratio of Akt2 was 6.35, higher than that of histopathological differentiation, another significant prognostic factor by multivariate analysis (Table III). These results indicate that Akt2 protein expression is a useful prognostic indicator in patients with HCC.

Akt is known to play an important role in various aspects of cancer progression, such as anti-apoptosis, angiogenesis, tumor invasion and metastasis (23-27). In this study, however, histopathological differentiation, and presence of portal tumor thrombus and multiple intrahepatic tumors were inversely related with Akt2 protein expression (Table II), suggesting that Akt2 may be a positive regulator of tumor differentiation in HCC. The exact causes for these controversial findings remain unknown at present. Akt2 may be involved in the early stages of hepatocarcinogenesis, as it was overexpressed in well-differentiated HCC, which is an early stage tumor, and may transform into a more undifferentiated type (33). For example, Akt2 is induced in colonic polyp-tissues at a very early stage in colon carcinogenesis (34). One possible mechanism for Akt2 overexpression in early HCC might be hypoxia, decreased tissue blood flow in well-differentiated HCC, which results from increased cellularity due to proliferating tumor cells (35). In response to hypoxia, Akt can be activated and induce expression of vascular endothelial factor (VEGF) and cyclooxygenase-2 (COX-2) via the transcription factors hypoxia-inducible factor or nuclear factor κB (NF-κB) (36,37). Both VEGF and COX-2 play important roles in angiogenesis, one of the representative features of more advanced, dedifferentiated HCC (38). Indeed, the expression of COX-2 and VEGF in HCC tissues decreases according to tumor dedifferentiation (39,40), a feature similar to that of Akt2 protein expression. Based on these findings, further studies are essential to elucidate the cause-effect relationship between Akt2 expression and malignant features of HCC.

In non-tumor tissues, Akt2 protein expression was more highly expressed in liver cirrhosis than in normal liver or chronic hepatitis (Table I), suggesting the relationship with progression of the underlying chronic liver disease. Akt, a direct target of PI-3 kinase, is activated following cell stimulation by various growth factors (9) and is considered to regulate intracellular processes including gene transcription of cell cycle-related proteins (41-43). Molecules involved in the G1-S phase of the cell cycle, such as cyclin D1, CDC25A, or inhibitors of the cell cycle, such as p21Cip1/WAF1 and P27KIP1, are all molecules acting down-stream of Akt. Overexpression of Akt2 can transform NIH 3T3 cells (44). HBx protein, a constitutive protein of hepatitis B virus, also interacts with Akt/PKB and stimulates its kinase activity, which in turn blocks apoptosis by transforming growth factor (TGF)-ß inhibition, caspase 3 inactivation or H-Ras activation (45-47). In addition, HBx, collaborating with other oncogenes, such as H-Ras, can transform immortalized rodent cells through activation of Akt (47). Therefore, it is possible that enhanced Akt activation may induce unrestricted cell proliferation leading to carcinogenesis in chronic liver disease.

In contrast to Akt2 protein expression, Akt1 protein was expressed only faintly in all tumor and non-tumor tissues, and there was no relationship with histopathological characteristics of HCC. Organ- and tissue-specific expression of Akt protein has been demonstrated. Indeed, Akt2 protein has been demonstrated to be a predominant isoform in ovarian cancer, colon cancer and pancreatic cancer, whereas Akt1 protein was predominantly detected in gastric cancer (10,11,29,34). Akt mRNA was expressed in both normal and tumor tissues of the colon, breast, prostate and lung (48). In this study, the expression of Akt2 protein was higher in both non-cancer and cancer tissues of the liver compared with that of Akt1 protein (Figs. 1 and 2). The reason for the difference between Akt1 and Akt2 protein expression is not yet well understood, and the degree of Akt immunostaining is not always correlated with its kinase activity. However, these findings suggest that Akt2 may play a more specific role than Akt1 in the regulation of carcinogenesis and progression of chronic liver disease.

In conclusion, we have demonstrated in the present study that expression of Akt2 in liver tissue was closely associated with both hepatocarcinogenesis and the post-operative prognosis of HCC.

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Clinical value of alterations in *p73* gene, related to *p53* at 1p36, in human hepatocellular carcinoma

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Abstract. A novel gene, *p73*, encoding a protein with significant homology to *p53* and showing functional similarities to *p53*, was identified at chromosome 1p36, at which tumor suppressor gene of hepatocellular carcinoma (HCC) is supposed to be. Involvement of *p73* in hepatocarcinogenesis is controversial and clinical value of *p73* alterations remains obscure. We investigated allelic status of *p73* in 63 patients with HCC. Loss of heterozygosity (LOH) in *p73* was analyzed by PCR-RFLP analysis. The results were compared with LOH on chromosome 1p surrounding *p73* locus, mutations of *p53* and *p73*, and clinicopathologic characteristics. LOH on *p73* was observed in 33% of informative tumors. LOH in *p73* was not always observed between the regions with LOH on chromosome 1p examined despite the significant association of LOH in *p73* with LOH on chromosome 1p. No mutations were detected in *p73*. Tumors with LOH in *p73* were more frequently detected in liver without cirrhosis than that with cirrhosis. There was no significant statistic association between the presence of LOH in *p73* and six different clinicopathologic characteristics such as age, sex, histological type, T stage, tumor diameter, and virus status. Disease-free survival rates of the patients with LOH in *p73* were significantly poorer than those without LOH in *p73*. Multivariate analysis indicated that presence of LOH in *p73* was independent prognostic factor in patients with HCC. These findings suggested that *p73* might play some role in tumor progression of HCC even though *p73* should not be considered a candidate gene on chromosome 1p of HCC and does not function as a tumor suppressor gene like *p53*. Identifying the patients with LOH of *p73* in tumors could be useful to predict early recurrence and to stratify the patients who need adjuvant therapy after operation.

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Abbreviations: HCC, human hepatocellular carcinoma; PCR, polymerase chain reaction; LOH, loss of heterozygosity; RFLP, restriction length polymorphism; RT-PCR, reverse transcription-PCR; SSCP, single strand conformation polymorphism; DFS, disease-free survival

Key words: *p73*, chromosome 1p, loss of heterozygosity, *p53*, hepatocellular carcinoma, polymerase chain reaction

Introduction

Hepatocellular carcinoma (HCC) is one of the most common human cancers in the world and it carries a poor prognosis (1). Various studies have confirmed its association with persistent hepatitis B or C viruses infection and other liver-damaging factors such as alcoholic cirrhosis and exposure to mycotoxins (1,2). Although each of these etiologic agents appears to predispose a hepatocyte to development of HCC, either directly or indirectly, the molecular mechanisms involved in hepatocarcinogenesis remain obscure. Tumorigenesis and progression of malignancy, however, are considered to be the multiple processes involving genetic alterations of several types, such as chromosomal deletions, chromosomal translocations, point mutations, and gene amplification (3). These changes can lead to activation of oncogenes or inactivation of tumor suppressor genes. There is evidence for widespread involvement of putative suppressor genes such as *p53* and Rb genes in development and progression of HCC (4,5), while a few alterations have been found in known oncogenes (6-9).

Somatic inactivation of tumor suppressor genes is usually achieved by intragenic mutations in one allele of the gene and by the loss of a chromosomal region spanning the second allele (3,10). Chromosomal analysis using microsatellite polymorphic markers has revealed loss of heterozygosity (LOH) of specific chromosomal regions in various types of cancers, e.g., colon, breast, and lung cancer (3,11-13). The mapping of regions with a high frequency of LOH has been critical for identifying tumor suppressor genes (3). In HCC, previous studies mainly relying upon either restriction fragment length polymorphism (RFLP) markers or microsatellite markers restricted to specific chromosome arms, have also defined a number of LOH on various chromosome arms (14-19). Especially on chromosome 1p, frequent LOHs in HCC were reported, suggesting the presence of tumor suppressor genes in this chromosomal regions (20,21). However, the gene responsible for this chromosomal region has not been identified yet.

Recently, a novel putative tumor suppressor gene, *p73*, mapped to the 1p36 region (22), in which frequent deletions were reported in patients with neuroblastoma (23,24) as well

as HCC. This gene encodes a protein with remarkable sequence similarity to the DNA-binding, transactivation, and oligomerization domains of *p53*, which is widely accepted to be involved in tumorigenesis and progression of a variety of malignant tumors including HCC (25,26). It also activates transcription of *p53* responsive genes and inhibit cell growth in a *p53*-like manner, by inducing apoptosis (27).

Despite several reports that demonstrated *p73* alterations in HCC, the involvement in hepatocarcinogenesis is still controversial due to the lack of the number of patients examined (28,29). Although increased expression of this gene in HCC is reported to be associated with poor patient prognosis in only one immunohistochemical study (30), clinical value of *p73* gene alterations remains obscure. In the present study, we examine LOH on *p73* including chromosome 1p region surrounding *p73* locus and mutations of *p73*, with reference to the *p53* mutation status, in 63 patients with HCC in order to elucidate the potential involvement of *p73* gene alterations in the development and progression of HCC.

Patients and methods

Patients and tissue preparation. Tumor and corresponding non-tumorous liver tissues from 63 HCC patients who underwent curative surgery at the Department of Surgery and Clinical Oncology, Graduate School of Medicine, Osaka University between June 1994 and August 1998, were collected with informed consent. This study included only patients from whom adequate tumor tissue was available for pathologic examination and molecular genetic analysis. Each tissue specimen was divided into two pieces at surgery. One for molecular analysis was immediately frozen by liquid nitrogen at the time of surgery and stored at -80°C until examination. Another was processed for pathologic examination. All 63 tumors were macroscopically or microscopically examined to determine location, size, extent and mode of cancer invasion, and metastasis to lymph nodes or distant organs according to the International Union Against Cancer tumor-nodes-metastasis (TNM) classification (31). Mean age of the patients was 61.5 years (range, 35-74 years). Fifty-two patients were men and 11 were women. Thirty-eight patients had HCV infection, 19 had HBV infection, and 6 had no HCV/HBV infection. Thirty-four patients had liver cirrhosis. Mean tumor diameter was 4.1 cm (range, 0.8-19.0 cm). Histologically, 9 tumors were classified as well-differentiated adenocarcinoma, 24 were moderately differentiated adenocarcinoma, and 30 were poorly differentiated adenocarcinoma. According to the TNM classification (31), 14 patients were T1N0M0 (stage I), 29 were T2N0M0 (stage II), 11 were T3N0M0 (stage III-A), and 9 were T4N0M0 (stage IV-A) (Table I). Patient follow-up was organized in Department of Surgery and Clinical Oncology, Graduate School of Medicine, Osaka University. All patients were followed until recurrence of disease or the end of the observation period (April 30, 2002). Mean follow-up period was 36.9±13.4 months.

A pair of tumor and the corresponding non-tumorous liver tissue was prepared for DNA and RNA analysis. Genomic DNA samples and total RNA were isolated using SepaGene (SankoJyunyaku, Tokyo, Japan) and TRIzol (Life Technologies, Grand island, NY) according to the manufacturer's protocols, respectively.

Table I. Summary of clinicopathologic characteristics of 63 patients.

Age (mean)	35-74 years (61.5)
Sex (Male/Female)	52/11
Histological type (well/mod/poor)	9/24/30
T stage (1/2/3/4)	14/29/11/9
Tumor diameter (mean)	0.8-19.0 cm (4.1)
HCV infection (+/-)	38/25
HBV infection (+/-)	19/44
Liver cirrhosis (+/-)[a]	34/26

[a]Three patients unknown.

Microsatellite polymorphism analysis. Genetic alterations on chromosome 1p in HCC were studied by microsatellite polymorphism analysis, as previously described (21,32). The CA-repeat microsatellite markers, D1S243, D1S228, D1S513, and D1S211 (33), which are located near the chromosomal region of *p73* were used. Primer sets labeled with fluorescent dye for PCR from Human MAPPAIRS (Research Genetics, Huntsville, AL) were used. PCR was performed in a 20 μl reaction mixture containing 0.05 μg of genomic DNA template, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphates, 0.2 mM of each primers, and one unit of AmpliTaq Gold (Perkin-Elmer, Foster city, CA) using Perkin-Elmer Cetus 9600 thermocycler (Perkin-Elmer Cetus). Reactions were carried for 30 cycles. Each cycle consisted of 45 sec at 95°C for denaturation, 1 min at 57°C for primer annealing, and 1 min at 72°C for primer extension. The first denaturation step was lengthened to 12 min for activation of AmpliTaq. The last extension step was lengthened to 7 min. Each PCR product (5 μl), mixed with 2 μl of blue dextran (80% formamide; 10 mM EDTA; 0.6% blue dextran), was heated at 95°C for 20 min and, then, electrophoresed in 6% denaturing polyacrylamide gel, containing 7.7 M Urea at 1500 V for 2 h. The image of fluorescent-labeled PCR products was scanned and acquired by a FMBIO II Multi-View (Takara, Shiga, Japan). When there was an absence, or a >50% reduction in intensity of one allele in tumor specimens comparing to the allele in normal liver, tumor was scored as exhibiting LOH, as previously described (21,32).

Examination of LOH at the p73 locus by PCR-RFLP analysis. PCR-RFLP analysis with restriction enzyme *Sty*I (Boehringer Mannheim, Mannheim, Germamy) was used to distinguish two distinct alleles, representing tightly linked polymorphisms in *p73* exon 2, as previously described (22). An allelic polymorphism consisting of a double nucleotide substitution (G→A) and (C→T) at position 4 and 14 of exon 2. Nested PCR

was performed under the following conditions. First PCR was performed with primer sets (CACCTGCTCCAGGG ATGC, AAAATAGAAGCGTCAGTC) (22) in a 25 µl reaction mixture containing 0.05 µg of genomic DNA template, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphates, 0.2 mM of each primers, and one unit of AmpliTaq Gold using Perkin-Elmer Cetus 9600 thermocycler. Reactions were carried for 35 cycles. Each cycle consisted of 30 sec at 95°C for denaturation, and 30 sec at 58°C for primer annealing. First denaturation step was lengthened to 9 min for activation of AmpliTaq. The last primer extension step was consisted of 10 min at 68°C. Second PCR step was performed with primer sets (CAGGCC CACTTGCCTGCC, CTGTCCCCAAGCTGATGA) (22) under the same condition of first PCR step using 0.5 µl of first PCR product. After second PCR, a specific 482-bp fragment was obtained. The PCR product (5 µl) was then digested with *Sty*I overnight. *Sty*I digestion of PCR products yields two smaller size fragments from AT allele-derived amplicons of 376 and 106 bp, respectively, whereas the GC allele-derived amplicons remain uncut, as previously described (22). The digestion results were analyzed on a 2% agarose gel.

PCR-SSCP analysis for the mutation screening of the p53 and p73. PCR-SSCP analysis was performed to screen the mutation of *p53* in exon 5-8 and mutation of *p73* in exon 1-13. Regarding PCR-SSCP analysis of the *p53*, primer pairs for *p53* were: (TTCAACTCTGTCTCCTTCCT, CAGCCC TGTCGTCTCTCCAG) for exon 5, (GCCTCTGATTCC TCACTGAT, TTAACCCCTCCTCCCAGAGA) for exon 6, (CTTGCCACAGGTCTCCCCAA, TGTGCAGGGTGGCAA GTGGC) for exon 7, (TTCCTTACTGCCTCTTGCTT, CGC TTCTTGTCCTGCTTGCT) for exon 8 (Nippongene, Tokyo, Japan). PCR was performed in a 20 µl reaction mixture containing 0.05 µg of genomic DNA template in the presence of a [^{32}P]-dCTP, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphates, 0.2 mM of each primers, and one unit of AmpliTaq Gold using Perkin-Elmer Cetus 9600 thermocycler. Reactions were carried for 35 cycles. Each cycle consisted of 30 sec at 95°C for denaturation, 30 sec at 64°C for primer annealing. The first denaturation step was lengthened to 12 min for activation of AmpliTaq. The last primer extension step was consisted of 10 min at 72°C, then, followed by electrophoretic separation on 5% non-denaturing polyacrylamide gel with 5% glycerol at room temperature. To screen mutation of *p73* gene, RT-PCR-SSCP analysis was performed as previously described (34). Reverse transcription (RT) was performed with 1 µg of total RNA and Reverse Transcription System (Promega, Madison, WI), according to the manufacturer's protocols. *p73* cDNA was amplified into 8 overlapping fragment (S1-AS1, S2-AS2, ..., S7-AS7, S8-AS8 primer sets) (34). The gel was stained by SYBR Green II RNA Gel Stain (FMC, Rockland, ME) and the image of PCR products was scanned and acquired by a FMBIO II Multi-View.

Statistical analysis. The clinicopathological characteristics of patients in relation to LOH in *p73* were analyzed by using Mann-Whitney U test or χ^2 test. The characteristics were also analyzed by multivariate analysis. Disease-free survival (DFS) curves were plotted according to the Kaplan-Meier method and were analyzed using the log-rank test. Statistical analysis was performed using Stat-View software version 5.0 (ABACUS, Berkeley, CA). A value of $p<0.05$ was considered statistically significant.

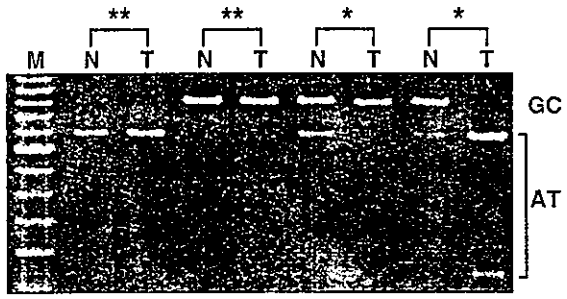

Figure 1. Representative result of LOH analysis in *p73* by PCR-RFLP analysis. Ethidium bromide staining comparing PCR-RFLP products with tumor (T) and matched normal liver (N) DNA samples. *Revealing LOH in T. **Homozygous samples. Lane M; size marker (50 bp DNA ladder).

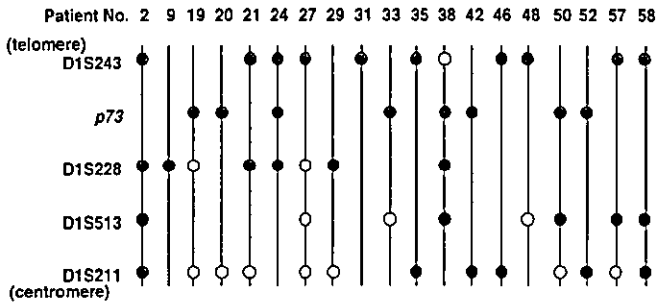

Figure 2. Schematic representation of partial deletions on chromosome 1p and *p73* in 19 HCC patients. Patient numbers are shown above, and the loci on the left. Closed circles, LOH; open circles, retained heterozygosity; blank space, uninformative.

Results

Twenty-four (38.1%) of 63 patients examined were GC/AT heterozygous for the exon 2 polymorphism and were informative for LOH analysis, while 35 patients (55.6%) were GC/GC homozygous and 4 (6.3%) were AT/AT homozygous. In 24 informative patients, LOH in *p73* was observed in 8 patients (33.3%) (Fig. 1).

All of 63 patients were informative for at least one microsatellite marker examined on chromosome 1p. More than one LOH on chromosome 1p were observed in 16 (25.4%) of 63 patients. The deletion mapping in 19 cases showing LOH on chromosome 1p or in *p73* is summarized in Fig. 2. Although the association of frequency of LOH in *p73* with LOH on chromosome 1p was statistically significant ($p<0.0005$; Table II), LOH in *p73* gene was not always observed between the regions with LOH on chromosome 1p examined (patient no. 9, 27, 29, 31, 33,38, 48, 50, 52 in Fig. 2).

To examine the possibility that *p73* function as a tumor suppressor, mutation in *p73* was screened using RT-PCR-

Table II. Association of LOH in *p73* with clinicopathologic variables.

Variables	LOH in p73 (+) (n=8)	LOH in p73 (-) (n=16)	p-value
Age (year)	61.0	61.8	0.85
Sex (male/female)	7/1	13/3	0.70
Histological type (well-mod/poor)	6/2	9/7	0.37
T stage (T1-2/T3-4)	6/2	10/6	0.54
Tumor diameter (≥2 cm/<2 cm)	1/7	6/10	0.20
LOH on chromosome 1p (+/-)	5/3	0/16	0.0004
HCV infection (+/-)	5/3	12/4	0.65
HBV infection (+/-)	2/6	4/12	>0.99
Liver cirrhosis (+/-)	1/7	11/5	0.027

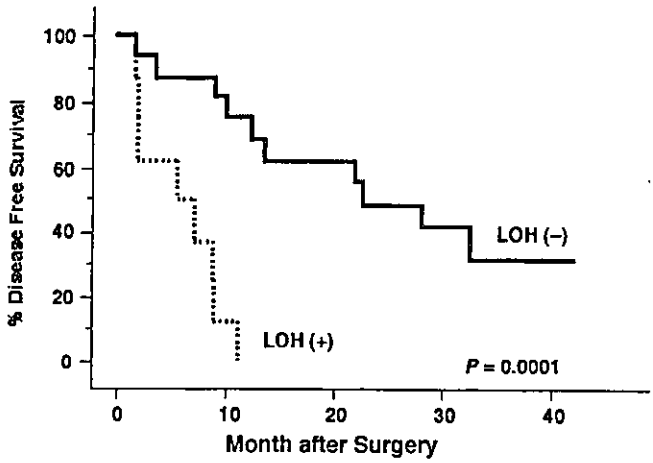

Figure 3. Comparison of disease-free survival in LOH (+) group vs. LOH (-) group. There was a significant difference in disease-free survival between the two groups (p=0.0001).

Table III. Relationship between LOH in *p73* and *p53* mutation.

		p53 mutation (-)	(%)	p53 mutation (+)	(%)
LOH in *p73*	(-)	12	(75)	4	(25)
	(+)	7	(87.5)	1	(12.5)
NI		32		7	
Total		51		12	

NI, not informative.

SSCP analysis. Several SSCP shifts were detected in a few exons in the pair of the tumor and normal liver DNA, suggesting that those should reflect sequence polymorphisms as previously described (22,34,35) (data not shown). Mutation screening in *p53* was also performed using PCR-SSCP analysis to examine the relationship between LOH on *p73* and *p53* mutations. Mutation in *p53* was observed in 12 (19.1%) among the 63 tumors. There were no mutations on *p53* in 7 of 8 patients with LOH in *p73*. Although LOH on *p73* was more frequent in tumors without *p53* mutations, statistical difference was not significant (Table III).

To study whether the alterations of *p73* are involved in hepatocarcinogenesis and progression, the relation between LOH in *p73* and clinicopathologic characteristics was examined. Although tumors with LOH in *p73* were more frequently detected in liver without cirrhosis than that with cirrhosis (p=0.027), there was no significant statistic association between the presence of LOH on *p73* and 6 different clinicopathologic features such as age, sex, histological type, T stage, tumor diameter, and virus status of 24 HCC patients informative for LOH on *p73* (Table II). Furthermore, the presence of LOH on chromosome 1p was significantly associated to LOH on *p73*. There were no significant statistic difference in DFS rate between the presence or the absence of LOH on chromosome 1p in all 63 patients (data not shown). However, DFS courve of the 8 patients with LOH on *p73* was significantly poorer than that of the 16 patients without LOH in *p73* (p=0.0001) (Fig. 3). Multivariate analysis using the Cox proportional hazard model indicated that presence of LOH in *p73*, tumor diameter and liver cirrhosis were independent prognostic factors in patients with HCC in spite of small number of patients (Table IV).

Discussion

p73 has recently been identified on chromosome 1p as a first family member of the *p53* tumor-suppressor gene (22). *p73* is predicted to encode a protein with significant amino acid sequence similarity to *p53* (22), and, like *p53*, can activate the transcription of *p53*-responsive genes, such as p21*waf1/cip1*, to inhibit cell growth in a *p53*-like manner by inducing apoptosis when over produced (27). Because of the close similarity between *p73* and *p53* in both structure and function, it was suggested that *p73* might also be a tumor suppressor (22). Furthermore, *p73* was shown to be located at the chromosomal localization 1p36, which is a region frequently deleted in a variety cancers including HCC (11,12,20,21). Up to this point, however, numerous studies have not discovered any coding region mutations of *p73*, an important feature of a tumor suppressor, in tumors including HCC (22,34-36). It is still controversial whether *p73* should be considered a tumor suppressor gene in hepatocarcinogenesis.

Several genetic changes which support the presence of tumor suppresser genes in HCC have been detected as loss of heterozygosity (37) and a genetic model of multistage carcinogenesis and progression has been proposed (38,39). In the present study, we first investigated whether or not *p73* may play a role as a tumor suppressor gene in HCC. Allelotyping analysis to detect LOH on *p73* and on chromosome 1p surrounding *p73* locus was performed on 63 surgically treated

Table IV. Multivariate analysis for disease recurrence.

Variable	Odds ratio (95% CI)	p-value
Age (year) ≥60 vs. <60	6.60 (0.66-65.80)	0.11
Sex Male vs. female	2.47 (0.37-16.55)	0.35
Histological type Well-mod vs. poor	1.68 (0.37-7.70)	0.51
T stage T1-2 vs. T3-4	2.80 (0.68-11.60)	0.16
Tumor diameter >2 cm vs. ≤2 cm	0.06 (0.004-0.832)	0.04
LOH in p73 (-) vs. (+)	15.00 (1.61-139.42)	0.02
LOH on chromosome 1p (-) vs. (+)	0.55 (0.06-5.29)	0.60
HCV infection (-) vs. (+)	0.67 (0.12-3.73)	0.64
HBV infection (-) vs. (+)	12.74 (0.87-186.83)	0.06
Liver cirrhosis (-) vs. (+)	0.04 (0.003-0.53)	0.01

95% CI, 95% confidence interval.

HCC tumors and corresponding non-cancerous liver tissues. LOH in *p73* was observed in 33% of tumors examined, a frequency higher than that in previous reports (28,29) and there was a significant correlation between the patients with LOH in *p73* and the patients with LOH on chromosome 1p (p=0.0004). Deletion map, however, showed that LOH in *p73* was not always detected between the regions with LOH on chromosome 1p examined. Although LOH on chromosome 1p have occurred in tumors at an early stage or with a well-differentiated HCC as well as in tumors at more advanced stages, and even in tumors <2 cm (21), LOH on *p73* was not associated with these features.

It is widely accepted that loss or inactivation of a tumor suppressor gene, *p53* contribute to the development of 50% of all human cancers (25,26). In HCC, *p53* mutations are frequently observed in moderately and poorly differentiated tumors (38), and, consequently, is to be associated with the grade of cancer cell atypia that advanced with tumor growth (39). However, frequency of *p53* aberration in HCC (4,5,38) were <30% as examined in the present study, and were lower than other malignant tumors (25,26), suggesting the presence of other suppressor genes except *p53*. Because of the remarkable sequence similarity to the DNA-binding transactivation, and oligomerization domains of *p53* (22), *p73* has been considered as a candidate suppressor gene in HCC. Although the present study showed that most of the patients with LOH in *p73* had no mutation in *p53* (87.5%), we did not detect any coding region mutations of *p73* in 63 tumors as previously reported in other types of tumors including HCC (22,28,34-36). These findings suggested that *p73* might not be a tumor suppressor gene on chromosome 1p. Otherwise, *p73* unlike *p53* may function in a different manner from Kunudson's two-hit theory (40), while mutation type of *p53* is loss of one allele and subtle structural change of the remaining allele (41,42). Further studies are needed to clarify the precise mechanism of *p73* on hepatocarcinogenesis.

The present study indicated LOH in *p73* as an indicator of early recurrence for patients with HCC treated by curative surgical resection. Patients with tumors which had LOH on *p73* had a statistically significant shorter disease-free survival than those without LOH on *p73*. Although cirrhotic status of liver is also one of important prognostic indicators for the recurrence of HCC after surgery (2), LOH on *p73* was frequently observed in patients without chirrosis. After comparing *p73* LOH status with other factors known to be of prognostic value in patients with surgically curative resection for HCC in a multivariate model, we identified LOH on *p73* as a new and independent marker for assessing the early recurrence of patients after curative resection. The previous study also identified the increased expression of p73 protein as an indicator of poor prognosis for patients with HCC treated by surgical tumor resection (30). However, the molecular mechanisms not only of the increased expression of *p73* in HCC but also of influence on poor prognosis remain obscure. Since overexpressed p73 protein is likely to be wild-type (30) also shown in the present study, LOH of *p73* may influence overexpression of p73 protein which could antagonize *p53* function increasing proliferation in tumor with a poor prognosis (30). Further work will have to clarify the relationship between LOH of *p73* and overexpression of *p73*.

In conclusion, we demonstrated that *p73* might play some role in tumor progression of HCC even though *p73* should not be considered a candidate gene on chromosome 1p of HCC and it does not function as a tumor suppressor gene like *p53*. Identifying the patients with LOH of *p73* in tumors could be useful to predict early recurrence and to stratify the patients who need adjuvant therapy after operation.

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Featured Article

Molecular Prediction of Response to 5-Fluorouracil and Interferon-α Combination Chemotherapy in Advanced Hepatocellular Carcinoma

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ABSTRACT

Purpose: **The prognosis of hepatocellular carcinoma (HCC) is very poor, particularly in patients with tumors that have invaded the major branches of the portal vein. Combination chemotherapy with intra-arterial 5-fluorouracil and subcutaneous interferon-α has shown promising results for such advanced HCC, but it is important to develop the ability to accurately predict chemotherapeutic responses.**

Experimental Design: **We analyzed the expression of 3,080 genes using a polymerase chain reaction-based array in 20 HCC patients who were treated with combination chemotherapy after reduction surgery. After unsupervised analyses, a supervised classification method for predicting chemotherapeutic responses was constructed. To minimize the number of predictive genes, we used a random permutation test to select only significant ($P < 0.01$) genes. A leave-one-out cross-validation confirmed the gene selection. We also prepared an additional 11 cases for validation of predictive performance.**

Results: **Hierarchical clustering analysis and principal component analysis with all 3,080 genes revealed distinct gene expression patterns in responders (those with complete response or partial response) and nonresponders (those with stable disease or progressive disease) to the combination chemotherapy. Using a weighted-voting classification method with either all genes or only significant genes as assessed by permutation testing, the objective responses to treatment were correctly predicted in 17 of 20 cases (accuracy, 85%; positive predictive value, 100%; negative predictive value, 80%). Moreover, patients in the validation dataset could be classified into two distinct prognostic groups using 63 predictive genes.**

Conclusions: **Molecular analysis of 63 genes can predict the response of patients with advanced HCC and major portal vein tumor thrombi to combination chemotherapy with 5-fluorouracil and interferon-α.**

INTRODUCTION

Hepatocellular carcinoma (HCC), the predominant histologic subtype of primary liver cancer, is one of the major causes of death from malignancy worldwide. Although recent progress in both diagnostic and surgical techniques has resulted in considerable improvement in the morbidity and mortality rates, the overall outcome remains far from satisfactory. The prognosis is miserable, particularly in patients with tumors that have invaded the major branch of the portal vein, and the median survival time is several months (1–3). Indeed, the 1-year survival rate was <50% even in resectable cases (3, 4). We and others have recently developed a new treatment regimen for such advanced HCC patients: combination chemotherapy of 5-fluorouracil (5-FU) and interferon (IFN)-α (5, 6). In follow-up studies, about 40% to 50% of patients showed promising responses to the therapy and lived >2 years. However, for patients who did not respond to this treatment, survival was too short to receive another type of treatment. Furthermore, this chemotherapy may have multiple adverse effects, including leukopenia, thrombocytopenia, and depression. Therefore, accurate prediction of chemosensitivity is desirable, not only so that nonresponding patients do not lose a limited chance to take advantage of other possible treatments, but also to eliminate suffering of these patients due to debilitating side effects. Unfortunately, there are currently no useful indicators to distinguish between patients who are likely to respond to this combination chemotherapy and patients who are not.

In this study, genes possessing the ability to predict patient responses to 5-FU and IFN-α combination chemotherapy were selected by gene expression profile analysis using adaptor-tagged competitive polymerase chain reaction (ATAC-PCR) technology, a polymerase chain reaction (PCR)-based array system (7). The

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performance of this prediction method was estimated with both a complete cross-validation and an external validation dataset.

MATERIALS AND METHODS

Tissues and Patients. Between August 1998 and January 2003, 20 HCC patients with multiple tumors spreading to bilateral lobes with tumor thrombi in the major branches of the portal vein underwent palliative surgery to reopen the portal flow and recover liver function. After obtaining informed consent, we collected HCC tissue from the main resected tumors and isolated the total RNA from these samples for PCR-based array experiments. All 20 patients had visible tumors in the remaining liver. The patients were then subjected to a treatment regimen of combination chemotherapy with 5-FU and IFN-α as described previously (5). Briefly, patients received continuous arterial infusion of 5-FU (450–500 mg/d) for the initial 2 weeks and subcutaneous IFN-α injection (5 million IU) 3 times per week for 4 weeks. The sizes of the remaining tumors in the liver, which are thought to be intrahepatic metastases from the resected primary tumor, were measured before and after treatment by either abdominal computed tomography scan or ultrasonography. The chemotherapeutic response was clinically evaluated according to the Eastern Cooperative Oncology Group (ECOG) criteria (8). In this study, responders were defined as patients with complete response (CR) or partial response (PR); nonresponders were defined as patients with stable disease or progressive disease (PD).

Furthermore, as an independent validation dataset, we collected liver tissue specimens and clinical data from 11 HCC patients who underwent hepatic resection of all visible tumors between October 1998 and October 2002. Although these patients had tumor(s) with major portal vein tumor thrombi, the area of spreading tumor(s) was limited to two segments of the liver. The treatment regimen of combination chemotherapy and the method of follow-up in the validation dataset were the same as the procedures used in the original dataset. All aspects of our study protocol were approved by the ethics committee of Osaka University Medical School.

Polymerase Chain Reaction-Based Array System. To select genes expressed in liver tissues, we constructed three cDNA libraries: one from a mixture of HCC and nontumorous livers, one from normal livers, and one from metastatic liver cancers, as described previously (9). We designed PCR primers for ATAC-PCR reactions for a total of 2,666 genes from these expressed sequence tag collections. In total, we prepared 3,080 primers for ATAC-PCR; this total includes an additional 414 genes established in previous literature. The specificity of this gene selection provides an advantage over more universal gene sets, such as those selected from the UniGene database, which include genes not expressed in liver tissues. The ATAC-PCR experimental procedure was performed as described previously (10). The complete list of genes and detailed protocols for the ATAC-PCR experimental procedure are available on our web site.[4]

Analysis of Polymerase Chain Reaction-Based Array Data. The relative expression level of each gene was calculated by calibration using a standard mixture of normal liver tissues, as described previously (11–15). After conversion to a logarithmic scale (base 2), the data matrix was normalized to a median of 0 by standardizing each sample.

As a supervised classification method, we adopted a weighted-voting (WV) algorithm generally used in gene expression profiling (16–19). Briefly, we calculated the signal to noise (S2N) ratio, $S_i = (\mu_R - \mu_N)/(\sigma_R + \sigma_N)$, where μ and σ represent the mean and SD of expression for responders and nonresponders, respectively. The magnitude of the gene vote (v_i) reflects the deviation of the test sample X_i value from the average of the two groups: $v_i = S_i \times (X_i - (\mu_R + \mu_N)/2)$. We summed the v_i values to obtain the total votes for a "good signature" group (V_G) and a "poor signature" group (V_P). The prediction strength is $(V_G - |V_P|)/(V_G + |V_P|)$, and we adopted a threshold of 0. If the strength is a positive number, the test sample belongs to the good signature group. If the strength is a negative number, the test sample belongs to the poor signature group. This model was evaluated by leave-one-out cross-validation (16–21), wherein one sample was randomly withheld, and the model was regenerated with the remaining samples and then used to predict the group of the withheld sample. This process was repeated until every sample was tested, and the cumulative accuracy was recorded. When an external validation dataset was used to evaluate this WV model, we used the S2N ratio (S_i) obtained from the original dataset. In calculating the weighted vote (v_i), the mean expression level of the entire validation dataset was substituted for the average of the two groups ($(\mu_R + \mu_N)/2$) in the original dataset.

Permutation testing, which involves randomly permuting group labels to determine gene-group correlations, was used to determine statistical significance (16–19). The original score, $So = |\mu_R - \mu_N|$, of each gene was calculated without permuting the labels (responder or nonresponder). The labels of all of the samples were then randomly permuted, and the scores were recalculated between two groups consisting of the new members. Repetition of this permutation 50,000 times provides a score number (No) larger than the original score (So). For each gene, the permutation P value was determined by $P = No/50,000$. To estimate the false discovery rate, which is the percentage of genes identified by chance (22), we calculated the Q value of every gene using downloaded software.[5] The Q value provides a measure of each feature's significance, automatically taking into account the problem of multiple testing (23).

Hierarchical cluster analysis using an unweighted pair group method using arithmetic mean method with Pearson's correlation and principal component analysis (PCA) was performed using GeneMaths 2.0 software. Other statistical analyses were performed using StatView 5.0J software. Correlations between the responses to our combination chemotherapy and clinicopathological parameters were evaluated by the χ^2 test with the Yates correction. Overall and disease-free survival rates were calculated using the Kaplan-Meier method. Differences in survival curves were estimated by the log-rank test.

[4] http://love2.aist-nara.ac.jp/laboratory/index_frame.html.

[5] http://faculty.washington.edu/~jstorey/qvalue.

Table 1 Characteristics of 20 patients

Case no.	Age (y)/sex	HBs Ag	HCV Ab	Child grade	Cirrhosis	AFP*	Edmondson classification	Main tumor size (cm)	Cycles of chemotherapy	Response†
1	74/M	−	+	A	Present	Low	3	3.0	3	CR
2	67/F	−	+	A	Present	High	3	9.2	3	CR
3	49/F	+	−	A	Absent	High	4	6.8	3	PD
4	47/M	+	−	A	Present	High	3	12.0	3	PD
5	51/F	−	−	B	Absent	High	3	15.0	3	PR
6	56/M	−	−	B	Present	Low	2	0.8	3	PD
7	69/M	−	+	A	Absent	High	3	8.0	3	CR
8	66/M	+	+	A	Present	High	3	4.0	3	CR
9	65/M	−	+	B	Present	High	3	1.3	2	PD
10	53/M	+	−	A	Present	High	3	17.0	3	PD
11	52/M	+	−	B	Absent	High	3	18.0	3	CR
12	66/M	−	−	B	Present	High	3	3.5	1	PD
13	39/M	+	−	B	Present	High	3	4.5	1	PD
14	56/M	−	+	A	Present	High	3	5.5	3	SD
15	67/M	−	+	A	Present	High	3	3.2	2	PD
16	47/M	+	−	A	Absent	High	3	13.0	3	PD
17	70/M	−	+	A	Absent	Low	3	9.4	3	CR
18	70/M	−	+	A	Absent	High	3	9.8	3	CR
19	71/M	−	+	A	Absent	High	3	9.0	3	PD
20	29/M	+	−	A	Absent	High	3	18.0	2	PD

Abbreviations: HBs Ag, hepatitis B surface antigen; HCV Ab, hepatitis C virus antibody; SD, stable disease.
* Divided into either low (≤50 ng/mL) or high (>50 ng/mL).
† Assessment based on ECOG criteria.

RESULTS

Patient Characteristics. The characteristics of the 20 HCC patients in the original dataset are shown in Table 1. All patients had multiple tumors spreading to bilateral lobes with tumor thrombi in the major branches of the portal vein and were treated with 5-FU and IFN-α combination chemotherapy after reduction surgery. Fifteen of the 20 patients were treated with three cycles of the above-mentioned regimen. The remaining five patients received only one or two cycles of 5-FU due to extensive progression of the tumors. According to the ECOG criteria of objective response, 8 patients (40%) were classified as responders, demonstrating either CR or PR for at least 4 weeks. The remaining 12 patients (60%) were classified as nonresponders, exhibiting either stable disease or PD. The clinicopathological characteristics of responders (CR and PR) and nonresponders (stable disease and PD) were compared by the χ^2 test (Table 2). There were no significant differences ($P < 0.05$) in any factor between the two response groups.

Unsupervised Analyses. We performed a hierarchical cluster analysis of the samples using all 3,080 genes. When the clinical samples were sorted on the basis of similarity in gene expression, the samples could be separated according to the response to 5-FU and IFN-α combination chemotherapy, *i.e.*, responders and nonresponders, with only a few exceptions (Fig. 1). Next, we applied PCA, a statistical method for reducing the number of data dimensions, to more simply present the relationships between the samples. On displaying the expression patterns of all 3,080 genes in three-dimensional space, we observed that most responders and nonresponders were located separately, indicating distinct gene expression patterns (Fig. 2).

Supervised Analyses. To construct a molecular prediction system, we used a supervised approach, a WV algorithm using S2N correlation ratios. The ability of this method to

Table 2 Clinicopathological characteristics between responders and nonresponders

Characteristics	Responders (n = 8)	Nonresponders (n = 12)	P
Age (y)			>0.05
<65	2	8	
≥65	6	4	
Sex			>0.05
Male	6	11	
Female	2	1	
HBs Ag			>0.05
Negative	6	6	
Positive	2	6	
HCV Ab			>0.05
Negative	2	8	
Positive	6	4	
Child grade			>0.05
A	6	8	
B	2	4	
Liver cirrhosis			>0.05
Absent	5	4	
Present	3	8	
AFP (ng/mL)			>0.05
≤ 50	2	1	
>50	6	11	
Histological grade			>0.05
Moderate	0	1	
Poor	8	10	
Undiff	0	1	
Tumor size (cm)			>0.05
≤5	2	5	
>5	6	7	

Abbreviations: Moderate, moderately differentiated hepatocellular carcinoma; Poor, poorly differentiated hepatocellular carcinoma; Undiff, undifferentiated carcinoma.

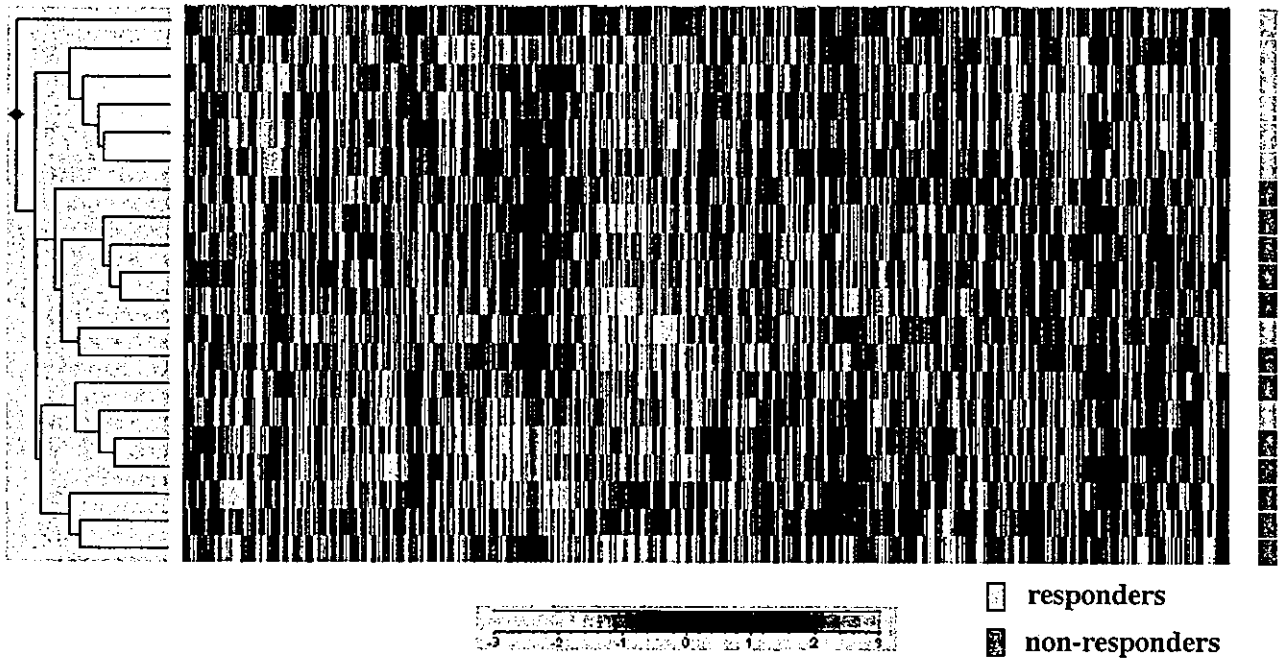

Fig. 1 Hierarchical clustering of 3,080 genes in 20 HCC cases. Each *column* represents a single gene, and each *row* represents a patient sample. The tissue type of each sample is designated by a *bar* at the *right*, and *green* and *red bars* indicate responders and nonresponders, respectively. The *color scale* at the *bottom* indicates the relative expression levels in terms of SDs from the mean.

predict patient responses to 5-FU and IFN-α combination chemotherapy was evaluated by leave-one-out cross-validation. In this first cross-validation analysis, we used all 3,080 genes to avoid any selection bias (Fig. 3*A*). The prediction accuracy of this WV method was 85.0%, and its 95% confidence interval ranged from 66.0% to 95.9%. The positive and negative predictive values were 100.0% and 80.0%, respectively.

These results demonstrate that this method provides a

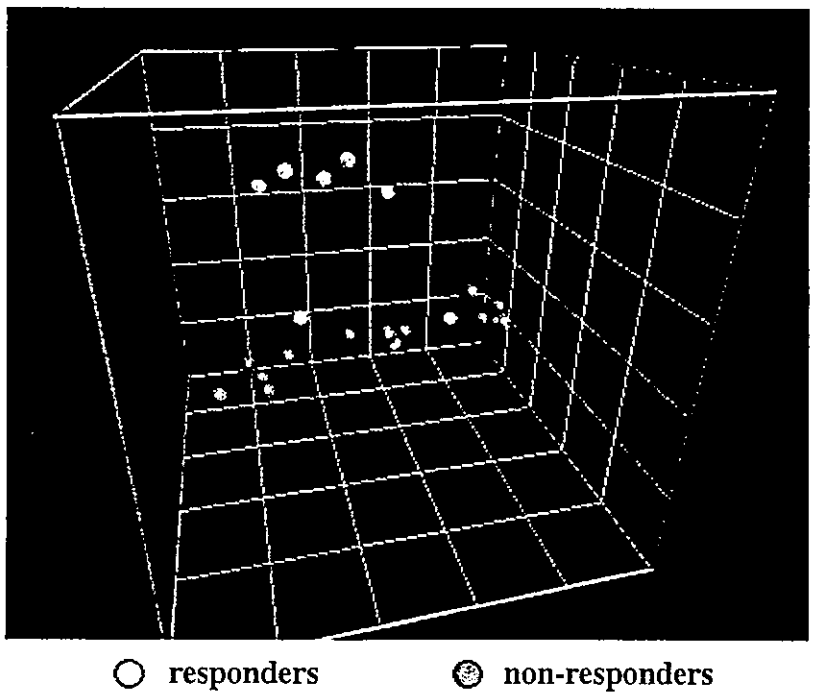

Fig. 2 PCA in 20 HCC cases. The variation is reduced to three-dimensional space; three components represent 37.0% of the total variance. Each *sphere* represents a single sample: *green* for a responder and *red* for a nonresponder.

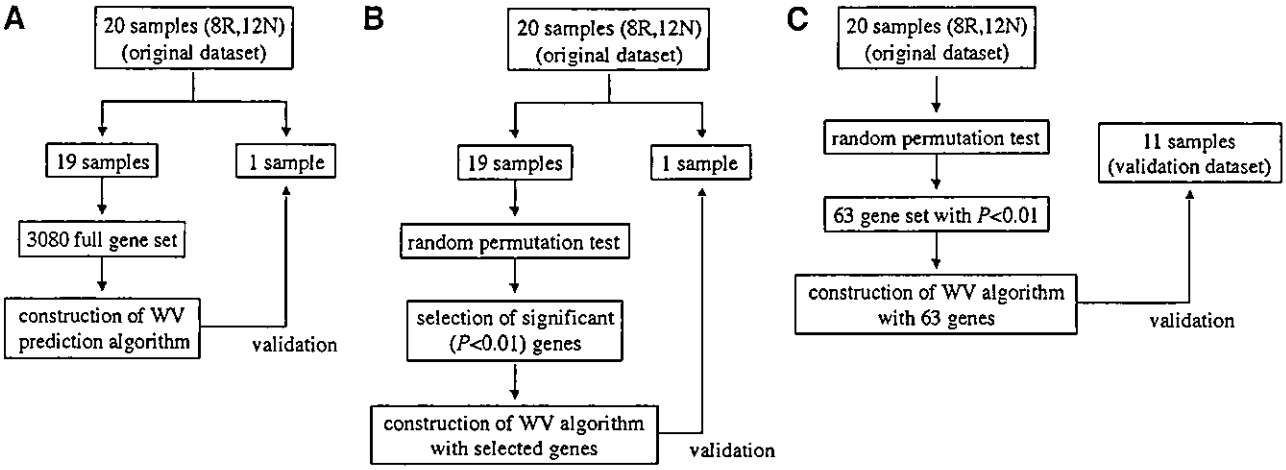

Fig. 3 Flow chart for selecting samples and gene set for analysis. *A*, the first approach, which used the original dataset and all genes. *B*, the second approach, which used the original dataset and only significant genes ($P < 0.01$). *C*, the third approach, which used the validation dataset and 63 significant genes selected with the original dataset.

valuable prediction of the chemotherapeutic response. To determine whether all 3,080 genes are necessary for prediction, we compared the prediction accuracy of a small number of genes to the prediction accuracy of all 3,080 genes. When the predictive genes were defined as genes with significant P values ($P < 0.01$) by the random permutation tests (Fig. 3*B*), the complete cross-validation without information leakage showed an identical performance with the initial cross-validation using all 3,080 genes. We therefore selected only 63 genes with significant P values between the 8 responder patients and the 12 nonresponder patients (Table 3). The median of their Q values, which is a measure in terms of the false discovery rate, was <25%. The expression patterns of these 63 genes exhibited distinct profiles between the two groups (Fig. 4).

To further evaluate our prediction system, we prepared an independent dataset, which consisted of 11 HCC patients with major portal vein tumor thrombi. Although these patients had no visible tumors after resection before combination chemotherapy, the risk of recurrence within the early postoperative period due to intrahepatic micrometastasis was very high (3, 4, 24). Moreover, in these advanced HCC cases with major portal vein tumor thrombi, the survival time was strongly correlated to the chemotherapeutic response. In fact, the median survival times of responders and nonresponders in the original dataset were 28 and 7 months, respectively, and the difference was statistically significant ($P = 0.002$). We therefore performed overall survival analysis and disease-free survival analysis instead of estimation to determine the prediction accuracy of objective response. Our prediction method using 63 genes classified patients into either a good signature group (those who were predicted to have good responses to this chemotherapy) or a poor signature group (those who were predicted to have poor responses; Fig. 3*C*). The overall survival rates were significantly different between these two predicted groups ($P = 0.001$; Fig. 5*A*). In addition, the good signature group had a distinctly better prognosis for disease-free survival than the poor signature group ($P = 0.002$; Fig. 5*B*).

DISCUSSION

HCC remains a major problem worldwide and appears to be increasing in developed Western countries (25). HCC is notorious for its poor prognosis because the overall 5-year survival rate after resection has remained as poor as 35% to 50%, even if curative surgery is performed (26–28). Moreover, once the tumor invades the major branch of the portal vein, the prognosis is extremely poor (1–4). Various complications including gastrointestinal bleeding, ascites, and hemorrhage from esophageal varices are provoked by stenosis of the portal vein. Until now, there has been no standard treatment for advanced HCC with portal tumor thrombi. However, we and others have recently developed a new combination chemotherapy regimen using intra-arterial 5-FU and subcutaneous IFN-α (5, 6), which has proven to be effective in about half of such advanced HCC patients. In this study, we have demonstrated that a molecular prediction method using expression data of 63 genes was able to distinguish patients who responded to this therapy from those who did not respond.

DNA microarray technology allows parallel expression analysis of thousands of genes to address complex questions in tumor biology. Many trials predicting the prognosis of various human malignancies have been reported using DNA microarrays (29–31). Moreover, a novel prediction method using gene expression profiling has recently been reported for the treatment of breast cancer patients with the taxanes (docetaxel and paclitaxel; ref. 21). Although DNA microarrays have contributed to such expression profiling studies to some degree, DNA microarrays can detect only a fraction of the changes in gene expression detectable by reverse transcription-PCR (32). We therefore performed a high-throughput quantitative PCR based on ATAC-PCR (7) to analyze the genetic differences in HCC. This assay requires smaller amounts of RNA than DNA microarray analysis. PCR-based analysis of selected genes costs much less than DNA microarrays, which are likely to require at least several hundred spotted genes for diagnosis. The aforementioned ben-

Table 3 The list of 63 predictive genes

P	Up/down	UniGene ID	Gene symbol	Gene definition
0.0001	Down	Hs.74088	*EGR3*	Early growth response 3
0.0002	Up	Hs.432818	*MFAP3*	Microfibrillar-associated protein 3
0.0002	Down	-	EST	DNC15 *Homo sapiens* cDNA
0.0003	Up	Hs.151738	*MMP9**	Matrix metalloproteinase 9
0.0005	Down	Hs.257697	*PDCD4*	Programmed cell death 4
0.0006	Up	Hs.337534	*EST*	*H. sapiens* clone 25061 mRNA sequence
0.0009	Down	Hs.293189	*FLJ37853*	*H. sapiens* cDNA FLJ37853 fis
0.0010	Down	Hs.6838	*ARHE*	Ras homolog gene family, member E
0.0010	Down	Hs.79078	*MAD2L1*	MAD2 mitotic arrest deficient-like 1
0.0011	Down	Hs.158244	*NMNAT2*	Nicotinamide nucleotide adenylyltransferase 2
0.0012	Down	Hs.175483	*FLJ22016*	*H. sapiens* cDNA: FLJ22016 fis
0.0012	Down	Hs.511400	*SND1*	Staphylococcal nuclease domain containing 1
0.0012	Up	Hs.301242	*MIDORI*	Myocytic induction/differentiation originator
0.0013	Down	Hs.471660	*FLJ36229*	*H. sapiens* cDNA FLJ36229 fis
0.0014	Down	Hs.81874	*MGST2*	Microsomal glutathione S-transferase 2
0.0015	Down	Hs.414156	*CTPS2*	CTP synthase II
0.0015	Up	Hs.118633	*OASL†*	2′-5′-oligoadenylate synthetase-like
0.0016	Down	Hs.406266	*HK2**	Hexokinase 2
0.0017	Down	Hs.75309	*EEF2*	Eukaryotic translation elongation factor 2
0.0019	Down	Hs.121150	*Taxilin*	Taxilin
0.0019	Down	Hs.67201	*NT5C*	5′, 3′-nucleotidase, cytosolic
0.0021	Down	Hs.417369	*CLNS1A*	Chloride channel, nucleotide-sensitive, 1A
0.0023	Down	Hs.13350	*UNC5B*	Unc-5 homolog B
0.0024	Down	Hs.345908	*TSC*	Hypothetical protein FLJ20607
0.0025	Down	Hs.2007	*TNFSF6**	Tumor necrosis factor (ligand) superfamily, member 6
0.0025	Up	Hs.440899	*KIAA1691*	KIAA1691 protein
0.0025	Down	Hs.143873	*S100A10*	S100 calcium binding protein A10
0.0026	Down	Hs.272630	*ATP6V1D*	ATPase, H+ transporting, lysosomal 34 kDa, V1 subunit D
0.0027	Down	Hs.413226	*ASB4*	Ankyrin repeat and SOCS box-containing 4
0.0027	Down	Hs.226391	*AGR2*	Anterior gradient 2 homolog
0.0027	Down	Hs.3610	*KIAA0205*	KIAA0205 gene product
0.0029	Down	Hs.335118	*JJAZ1*	Joined to JAZF1
0.0031	Down	Hs.12840	*LOC285859*	Hypothetical protein LOC285859
0.0031	Down	Hs.82324	*KIAA0157*	KIAA0157 protein
0.0031	Up	Hs.421825	*PPA2*	Inorganic pyrophosphatase 2
0.0036	Up	Hs.143773	*eIF3k*	Eukaryotic translation initiation factor 3 subunit k
0.0036	Up	Hs.380877	*SGKL*	Serum/glucocorticoid regulated kinase-like
0.0038	Down	Hs.387183	*BLMH*	Bleomycin hydrolase
0.0038	Up	Hs.114648	*ERG-1*	Estrogen-regulated gene 1
0.0042	Down	Hs.34012	*BRCA2*	Breast cancer 2, early onset
0.0048	Down	Hs.512628	*PPP2R2A*	Protein phosphatase 2, regulatory subunit B, α isoform
0.0051	Up	Hs.377975	*IGKC*	Immunoglobulin κ constant
0.0053	Down	Hs.435765	*ENPEP†*	Glutamyl aminopeptidase
0.0055	Down	Hs.356076	*BIRC4†*	Baculoviral IAP repeat-containing 4
0.0055	Down	Hs.220745	*FLJ10244*	Ral-A exchange factor RalGPS2
0.0060	Down	Hs.376531	*LOC283167*	*H. sapiens* LOC283167
0.0061	Down	Hs.128410	*GLS*	Glutaminase
0.0061	Down	Hs.356876	*GPR125*	G protein-coupled receptor 125
0.0065	Down	Hs.250696	*KDELR3*	KDEL endoplasmic reticulum protein retention receptor 3
0.0066	Down	Hs.10949	*ERO1L*	ERO1-like
0.0068	Down	Hs.380446	*LENG8*	Leukocyte receptor cluster member 8
0.0070	Up	Hs.22129	*DJ1042K10.2*	Hypothetical protein DJ1042K10.2
0.0070	Down	Hs.93177	*IFNB1*	Interferon β1, fibroblast
0.0072	Down	Hs.389461	*SMAP-1*	Smooth muscle cell-associated protein 1
0.0074	Up	Hs.512654	*SET07*	PR/SET domain containing protein 07
0.0080	Down	Hs.432574	*POLR2H*	Polymerase II polypeptide H
0.0080	Down	Hs.183986	*PVRL2*	Poliovirus receptor-related 2
0.0088	Down	Hs.406351	*EST*	*H. sapiens* clone IMAGE:120162 mRNA sequence
0.0089	Down	Hs.2533	*ALDH9A1*	Aldehyde dehydrogenase 9 family, member A1
0.0090	Down	Hs.282410	*CALM1†*	Calmodulin 1
0.0091	Down	Hs.37288	*NR1D2*	Nuclear receptor subfamily 1, group D, member 2
0.0096	Down	Hs.11355	*TMPO*	Thymopoietin
0.0098	Up	Hs.299208	*COQ3*	Coenzyme Q3 homolog, methyltransferase

NOTE. *P* values were calculated by random permutation test. Up-regulation and down-regulation were defined as expression in responders compared with nonresponders.

* Genes previously reported to be associated with sensitivity to 5-FU.

† Genes previously reported to be associated with sensitivity to IFN.

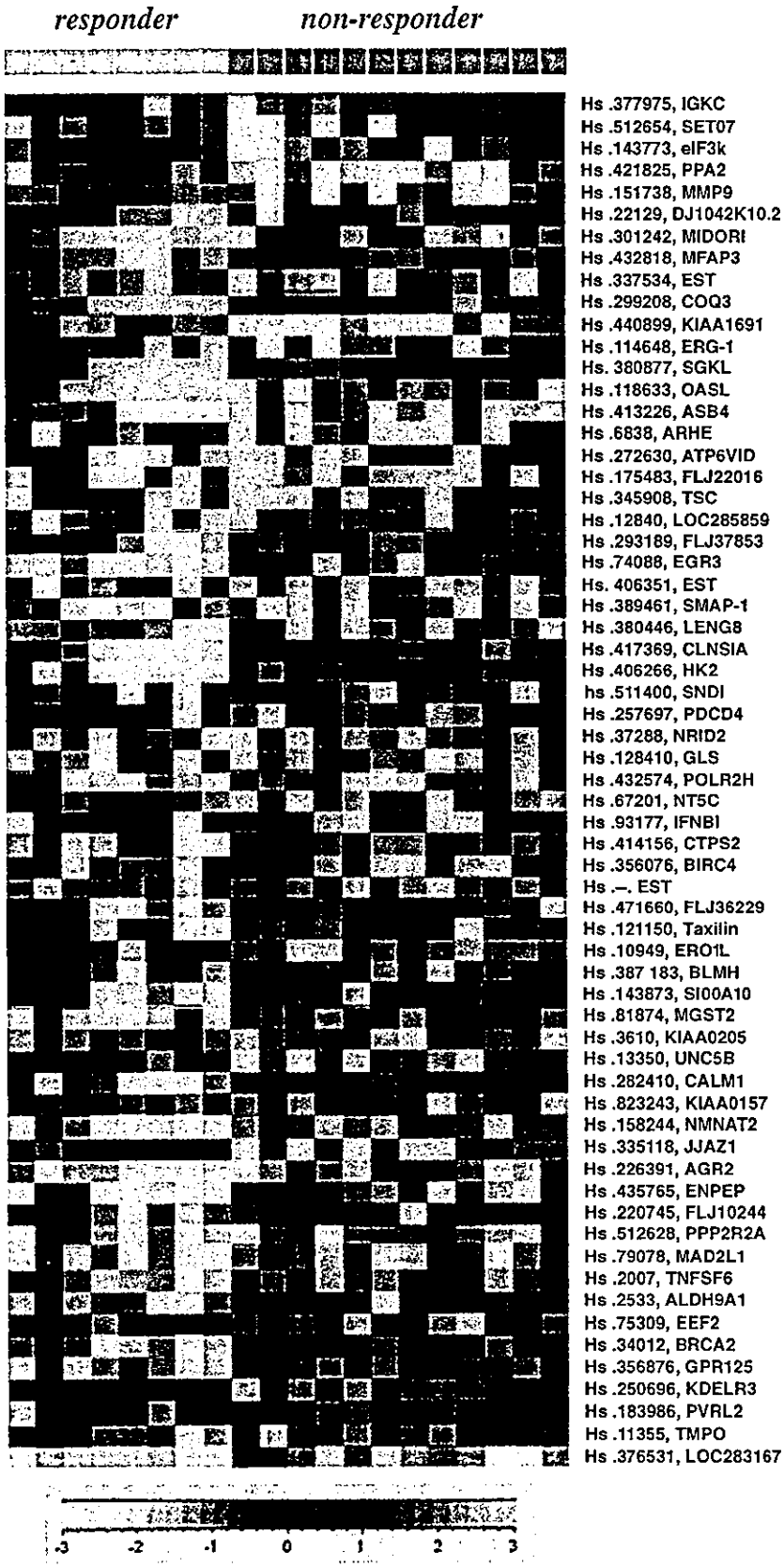

Fig. 4 The expression pattern of the 63 predictive genes in 20 HCC cases. Each *column* represents a patient sample, and each *row* represents a single gene.

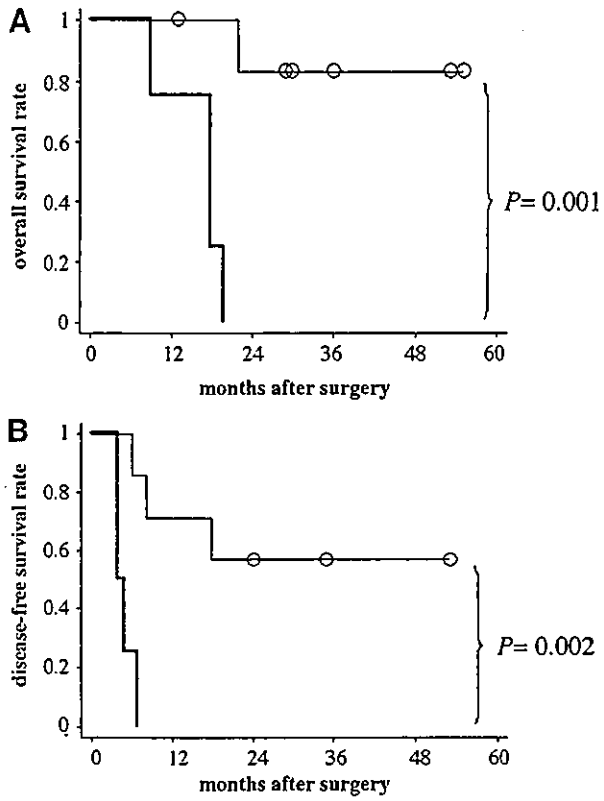

Fig. 5 Overall survival curves (*A*) and disease-free survival curves (*B*) calculated using the Kaplan-Meier method for the validation dataset. Differences in survival curves were estimated by the log-rank test.

efits and the strength of this system for cancer research, established in previous reports on HCC, breast, and colorectal cancers make this technique a powerful means to obtain a better understanding of the molecular characteristics of cancers (11–15).

In the unsupervised analyses with all examined genes, the difference of expression patterns between responders (CR and PR) and nonresponders (stable disease and PD) was clear. Of course, it was controversial how to divide two response groups. However, the response to this regimen with 5-FU and IFN-α was usually typical; that is, PR or stable disease cases were rare, as shown in Table 1. Clinically, radiologically, and biochemically, the PR case in this study revealed a near CR, and the stable disease case was similar to PD cases. We therefore classified the PR patient as a responder and the stable disease patient as a nonresponder due to the conventional grouping method (33, 34). In supervised analyses, we selected 63 significant genes by a random permutation test to minimize the number of predictive genes. These 63 predictive genes included some genes that have previously been reported to be associated with sensitivity to 5-FU or IFN. For example, a gene related to 5-FU sensitivity is tumor necrosis factor ligand superfamily member 6 (TNFSF6), also called FAS (CD95) ligand, which plays a major role in the induction of apoptosis in response to a variety of extracellular signals, including anticancer drugs. In hepatoma cells or human breast cancer, up-regulation of TNFSF6 was associated with poor sensitivity to 5-FU chemotherapy (35, 36). Matrix metalloproteinase 9 (MMP9), a member of the secreted zinc metalloprotease group, is also inhibited by 5-FU treatment (37). Hexokinase 2 (HK2) plays an important role in intracellular glucose metabolism and is strongly associated with the metabolism of 2′-fluoro-2′-deoxy-D-mannose, which has been correlated to 5-FU response (38). A gene involved in sensitivity to IFN-α is OASL, a member of the 2′-5′-oligoadenylate synthetase gene families. OASL plays an important role in the antiviral effects of IFN and is involved in apoptosis and control of cellular growth (39, 40). Baculoviral IAP repeat-containing 4 (BIRC4; XIAP), encodes a protein that significantly inhibits apoptosis. IFN inhibited BIRC4 function by inducing XIAP associated factor-1 (XAF-1; ref. 41). Glutamyl aminopeptidase is a differentiation-related kidney glycoprotein of 160 kDa (gp160), and the absence of glutamyl aminopeptidase expression was predictive of IFN-α sensitivity in renal cell carcinoma (42). Calmodulin-1 (CALM1) plays a role in growth and the cell cycle and in signal transduction. Calmodulin inhibitors affect not only the uptake and processing of IFN inducers but also the release of IFN from induced cells (43). Our selection approach to these predictive genes was confirmed with both a complete cross-validation and an external validation dataset.

In our univariate analysis with clinicopathological factors, there was no significant indicator that distinguished responders and nonresponders. Patt *et al.* (6) reported that low levels (≤50 ng/mL) of serum α-fetoprotein (AFP) were a predictor of response to 5-FU and IFN-α combination chemotherapy, but the serum AFP level was not a significant indicator of chemotherapeutic response in our study. Because we treated only extremely advanced HCC patients with major portal vein tumor thrombi with this combination therapy, only 3 of the 20 patients showed low levels of serum AFP. It may be too early to make conclusions regarding this factor, but we believe that another appropriate method will be required to more accurately predict the response to this treatment.

Indeed, this combination chemotherapy of 5-FU and IFN-α is promising for advanced HCC, but we need to consider the side effects. IFN-α and/or 5-FU sometimes induce severe adverse effects, including myelosuppression, fever, and depression (44). In particular, adverse myelosuppression is an important factor in HCC cases not only because thrombocytopenia and/or leukopenia are frequently present before chemotherapy, but also because treatment often has to be discontinued due to these side effects. Moreover, the survival of patients with such extremely advanced HCC was too short to allow a secondary treatment (1–4). Therefore, accurate prediction of sensitivity to the first-line chemotherapy is necessary for advanced HCC patients so that they do not lose their limited chance to receive treatment and the patients who will not respond to the therapy can be protected from debilitating side effects.

These results illustrate the potential of biological technology to advance diagnostic methods, allowing physicians to plan beyond empirical results toward a more molecularly well-defined, personalized therapy. Particularly for patients with poor prognosis, such as advanced HCC patients, accurate prediction of chemotherapeutic responses before treatment is necessary. Because our molecular prediction system involves only a small